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Exhibit A

Comparison of the acid stability of azithromycin and erythromycin A

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In acidic aqueous media, erythromycin A is rapidly degraded via intramolecular dehydration to form erythromycin-6,9-hemiketal and then anhydroerythromycin, both of which possess little antimicrobial activity. Azithromycin, a new azalide antibiotic, has a methyl-substituted nitrogen in place of the carbonyl at the 9a position of the aglycone ring, thus blocking the internal dehydration pathway. As a result, azithromycin decomposition occurs primarily via acid-catalysed hydrolysis of the ether bond to the neutral cladinose sugar.

Rate constants and the time for 10% decay (T_{10}) were determined for both azithromycin and erythromycin A at pH 2 using various levels of acetonitrile cosolvent and constant ionic strength. Semi-log plots of the decay rate constants versus the reciprocal of the solution dielectric constants were used to extrapolate to totally aqueous conditions. In solution at 37°C and pH 2 with ionic strength $\mu = 0.02$, azithromycin was degraded with a T_{10} of 20·1 min while erythromycin underwent 10% decay in only 3·7 sec. The activation energy for hydrolysis of the ether bond connecting cladinose to azithromycin was 25·3 kcal·mol^{-1} while the internal dehydration reaction of erythromycin had an activation energy of 15·6 kcal/mol.

A solution stability profile was generated for azithromycin over the pH range of 1·0 to 4·1 at 30°C. Stability was found to improve ten-fold for each unit increase in pH.

Introduction

In aqueous acidic media, erythromycin is known to undergo rapid decomposition via the pathway shown in Figure 1. The rate limiting first step of this reaction is reported to be subject to both general and specific acid catalysis with a stability maximum in the range of pH 7·5 to 8·5 (Kondrat'eva & Bruns, 1962; Kurath *et al.*, 1971; Amer & Takla, 1978; Atkins, Herbert & Jones, 1986). This decomposition reaction is also catalysed by cupric, ferrous and aluminium cations (Amer & Takla, 1978). Both the 6,9-hemiketal and anhydroerythromycin are known to be inactive against Gram-positive bacteria normally affected by erythromycin (Stephens & Conine, 1959).

Azithromycin (CP-62,993, XZ-450), the prototype azalide antibiotic, differs structurally from erythromycin A by replacing the 9a carbonyl in the aglycone ring with a methyl substituted nitrogen, as well as expansion of the ring to 15 members (Bright *et al.*, 1988). This structural difference blocks the internal reaction to form the hemiketal, leaving acid hydrolysis of the ether bond to the neutral cladinose sugar as the main decomposition pathway (Figure 2). Data from the erythromycin literature suggest that the azithromycin decay product without the cladinose sugar, CP-66,458, will lack bioactivity (LeMahieu *et al.*, 1974; Kibwage *et al.*, 1987).

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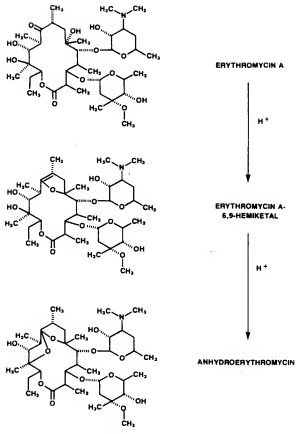

Figure 1. Decomposition pathway for erythromycin in aqueous acidic medium.

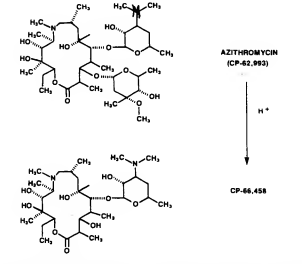

Figure 2. Decomposition pathway for the azalide, azithromycin, in aqueous acidic medium.

The objective of this study was to examine the stability of azithromycin in acidic solution and compare this with erythromycin stability.

Materials and methods

Antibiotics

Azithromycin was prepared at Pfizer Central Research, Groton, CT. Erythromycin A was obtained from The Upjohn Company, Kalamazoo, MI. Oleandomycin was obtained from Pfizer Taito Co. Ltd, Nagoya, Japan. All degradation products were isolated on site and identification was verified by NMR and mass spectroscopy, using published values (Kurath & Egan, 1971; Kurath *et al.*, 1971; Kibwage *et al.*, 1987; Bright *et al.*, 1988).

HPLC assay

A reverse-phase HPLC assay was developed for each antibiotic using an alkaline mobile phase and a polystyrene divinylbenzene stationary phase (Table 1). The equipment for this assay consisted of a M6000A pump and a Model 441 detector, λ = 214 nm, (Waters Chromatography Division, Millipore Corporation, Milford, MA), a PRP-1 analytical column, 5 μ, 4·1 mm ID × 15 cm L (Hamilton Company, Reno, NV), and a HP3390A integrator (Hewlett Packard Co., Avondale, PA). The temperature of the column was maintained at 50°C with a Model 725-1010 column heater (Rainin Instrument Co., Woburn, MA). The flow rate was maintained at 1 ml/min. The injection volume was 50 μl. All reagents in these assays were used as received (Fisher Scientific and Sigma Chemical Co.) without further purification.

These HPLC assays were derived by slight modifications of several published assays for erythromycin (Kibwage *et al.*, 1985; Nilsson, Walldorf & Paulsen, 1987). In each case, the intact parent compound was clearly separated from the decay products, so that consecutive reaction products could be monitored if necessary. The sensitivity of the assay for each compound was 10 mg/l and linear responses were obtained up to 2 g/l.

Table 1. Reverse-phase HPLC assay conditions used to study the acid decay of erythromycin A and azithromycin

Mobile phase: (% v/v)	
erythromycin A	azithromycin
20·0% TRIS 0·02 M	10% TRIS 0·02 M
27·5% TBAOH 0·02 M	20% TBAOH 0·02 M
52·5% Acetonitrile	10% Isopropanol
	60% Acetonitrile

Each mobile phase was adjusted to a final pH10 with concentrated phosphoric acid.

TRIS, tromethamine; TBAOH, tetrabutylammonium hydroxide, 40% solution.

Methods

Aqueous buffers for this study consisted primarily of 0·01 N HCl (pH 2) with sufficient NaCl to bring all the buffers to constant ionic strength (μ = 0·02). Buffers at higher pH values were prepared from mixtures of 0·01 M citric acid and 0·02 M sodium phosphate with the ionic strength adjusted to μ = 0·02 with NaCl.

Since the decay of erythromycin at pH 2 is known to be very rapid, a stock solution in acetonitrile was used to give a precise kinetic time zero at the time of drug addition. Portions of acetonitrile (5–25% v/v) were pre-mixed with the buffers to eliminate thermal changes due to heat of mixing upon addition of the stock antibiotic solution. Acetonitrile was chosen for the cosolvent because of its known linear change in solution polarity with changing cosolvent levels (Cunningham, Vidulich & Kay, 1967).

Before each experiment, the aqueous buffers and stock solutions of antibiotic in acetonitrile were pre-equilibrated at the desired temperature. At time zero, 10 ml of the stock antibiotic solution was added to 90 ml of buffer solution which was being mixed vigorously. This addition of acetonitrile stock drug solution brought the final solution to 15, 25 or 33·3% v/v cosolvent. After the antibiotic was added to the buffer, samples were withdrawn at various times, neutralized with an equal volume of mobile phase and then quantitatively assayed by HPLC. The erythromycin studies used acid stable oleandomycin as an internal marker to correct for mass balance during the rapid neutralization step (Nilsson *et al.*, 1987).

In all cases, first order decay kinetics were observed and monitored through at least two half-lives. Decay rate constants (k) were determined by linear regression analysis and the time for ten percent decomposition ($T_{1\,10}$) calculated from these decay rates. To construct a pH stability profile, a solution of pH 1 was prepared using 0·1 N HCl which had an ionic strength of μ = 0·105 without addition of NaCl, a higher ionic strength than solutions at pH 2–4. For neutralization of samples from this acidic buffer, three parts of neutralization medium, consisting of 66% v/v acetonitrile and 34% 0·2 M tromethamine, were mixed with one part of sample.

Results

Erythromycin A

Figure 3 shows the effect of acetonitrile cosolvent, plotted as the reciprocal of the solution dielectric (ε) constant, on the decay of erythromycin A at pH 2 and at three temperatures. The lower values on the x-axis ($1/\varepsilon$) correspond to higher levels of cosolvent. Extrapolation of the line of best fit obtained by linear regression to the dielectric constant for water of 78·35 ($1/\varepsilon$ = 0·0127) gave rate constants and $T_{1\,10}$

Table II. First order decay rate constants and $T_{1\,10}$ values for erythromycin A at pH 2 and ionic strength μ=0·02 in the absence of acetonitrile cosolvent

Temperature (°C)	k (min^{-1})	$T_{1\,10}$ (min)
30	0·94	0·1120 (6·72 sec)
40	2·29	0·0460 (2·76 sec)
50	4·67	0·0226 (1·36 sec)

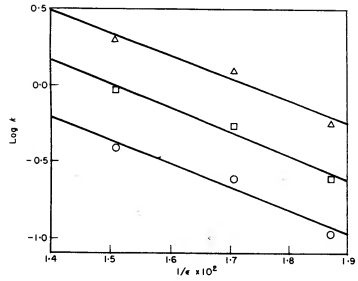

Figure 3. Effect of temperature and acetonitrile cosolvent level on erythromycin decay rate constants (k) in aqueous acidic (pH 2) media. △, 50°C; □, 40°C; ○, 30°C.

values for a totally aqueous medium (Table II). The presence of the internal marker oleandomycin did not alter the observed decay kinetics. Figure 4 shows an Arrhenius plot of temperature versus decay rate constants which, when analysed by linear regression, gave an activation energy of 15·6 kcal/mol. From Figure 4, $T_{1/10}$ was determined to be 0·0616 min (3·7 sec) for acid decay of erythromycin A at 37°C.

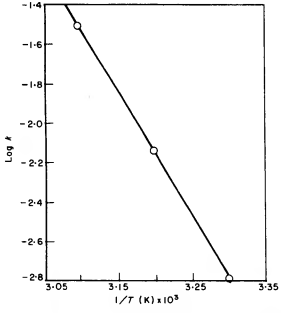

Figure 4. Arrhenius plot of temperature versus first order decay of erythromycin A at pH 2. E_{act} = 15·6 kcal/mol.

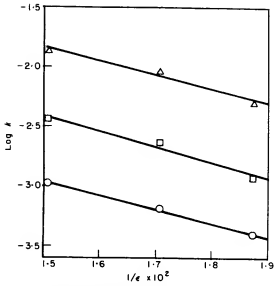

Figure 5. Effect of temperature and acetonitrile cosolvent on azithromycin decay rate constants in aqueous acidic (pH 2) media. △, 50°C; □, 40°C; ○, 30°C.

Azithromycin

Figure 5 shows the effect of cosolvent on the acidic decay of azithromycin at three temperatures. The decay rate constants and $T_{1/10}$ values were determined by extrapolating to a totally aqueous environment (Table III). As azithromycin was relatively stable in this acidic medium, a similar set of decomposition reactions was conducted in media without acetonitrile cosolvent and the results are shown as 'actual' data.

An Arrhenius plot of temperature versus first order decay rate constant data for azithromycin gave an activation energy of 25·3 kcal/mol (Figure 6). The $T_{1/10}$ of azithromycin at 37°C was 20·1 min. Similar results were obtained for acidic (pH 2, 37°C) hydrolytic cleavage of cladinose from anhydroerythromycin (activation energy = 28·8 kcal/mol; $T_{1/10}$ = 16·7 min).

Figure 7 shows an expanded pH-stability profile for azithromycin at 30 C in the absence of cosolvent. Although most of these data were generated with solutions at constant ionic strength (μ = 0·02), the highly acidic solution at pH 1 also had a higher ionic strength, μ = 0·105. The data in Figure 7 indicate a ten-fold increase in stability for each unit increase in pH.

Table III. First order decay rate constants and $T_{1/10}$ values for azithromycin at pH2 and ionic strength μ = 0·02 and in the absence of acetonitrile cosolvent

Temperature (°C)	Extrapolated data k (min^{-1})	Extrapolated data $T_{1/10}$ (min)	Actual $T_{1/10}$ (min)
30	$2·028 \times 10^{-3}$	51·96	64·9
40	$7·787 \times 10^{-3}$	13·53	14·5
50	$2·726 \times 10^{-2}$	3·87	3·34

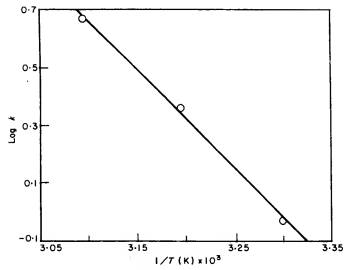

Figure 6. Arrhenius plot of temperature versus first order decay of azithromycin at pH 2. E_{act} = 25·3 kcal/mol.

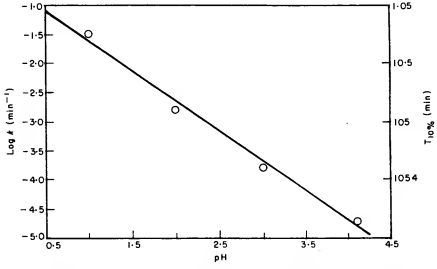

Figure 7. pH-stability profile for azithromycin at 30 C in the absence of cosolvent.

Discussion

Kondrat'eva & Bruns (1962) generated an exponential equation to describe the pH stability profile for erythromycin from pH 3 to 12 and from 0 to 70°C in media without acetonitrile cosolvent. This equation was used to compute the kinetics for decay at 37°C and pH 2, resulting in a $T_{1\,10}$ prediction of 3·3 sec with an activation energy of 19·1 kcal/mol. These values are close to the values observed in the present study ($T_{1\,10}$ = 3·7 sec and activation energy = 15·6 kcal/mol).

A reduction in the reaction rates with increasing acetonitrile cosolvent level is consistent with formation of a solvent shield around the attacking proton (Laidler, 1965). Further proof of this mechanism will require additional studies with cosolvents varying in dielectric constant and molecular size.

The acid-catalysed hydrolysis of the ether bond connecting the 15-member aglycone ring of azithromycin to the neutral cladinose sugar is consistant with published information concerning ether bonds (Burwell, 1954). The mechanism proposed for this reaction starts with proton attack on the ether oxygen followed by cleavage to the respective alcohols. The effect of cosolvent on creating a solvent shield around the attacking proton could account for the observed decrease in decay rate with increasing cosolvent level. In addition, a linear pH-stability profile in which each unit change in pH results in a ten-fold change in decay rate (Figure 7) is consistent with this mechanism. As expected, the acid decay product was found to have no in-vitro activity against micro-organisms normally sensitive to erythromycin and azithromycin (Girard, A. E., personal communication).

The pharmaceutical significance of this study lies in the difference in the chemical stability of the two antibiotics in media similar to gastric fluid. The acid instability of erythromycin requires enteric formulations that protect the antibiotic from stomach acidity. As macrolides are thought to be absorbed primarily in the upper duodenum, the enteric polymer coating must protect the compound in the stomach, but then dissolve quickly at pH 4–5 to deliver the drug to the site of maximum absorption (Watanabe *et al.*, 1978). The inherent acid stability of azithromycin suggests that an enteric formulation may not be necessary. The greater acid stability of azithromycin may account for higher plasma concentrations than are obtained with oral erythromycin (Girard *et al.*, 1987).

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